

Induction of anti-phospholipid syndrome in naive mice with mouse lupus monoclonal and human polyclonal anti-cardiolipin antibodies

(autoimmunity/autoantibodies/systemic lupus erythematosus/lupus anticoagulant)

M. BLANK*, J. COHEN*, V. TODER†, AND Y. SHOENFELD*

*Research Unit of Autoimmune Diseases, and †Departments of Medicine 'B', and Embryology, Sheba Medical Center, Tel-Hashomer, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv 52621, Israel

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ABSTRACT The primary anti-phospholipid syndrome is characterized by recurrent venous and arterial thromboembolic phenomena, recurrent fetal loss, thrombocytopenia, and serological evidence of anti-cardiolipin (aCL) antibodies or/and the presence of lupus anticoagulant (prolonged activated partial thromboplastin time). The exact role of aCL antibodies in pathogenesis is not clear and the mechanism by which the antibodies may induce the various manifestations is unknown. In the current study we evaluated the effect of passive transfer of aCL antibodies (to the tail vein of naive mice) on fecundity, fetal loss (fetal resorption), and the weight of embryos and placentae. Two types of aCL antibodies were employed: (i) mouse monoclonal aCL antibodies derived from a BALB/c mouse in which experimental systemic lupus erythematosus was induced by a pathogenic idotype (idiotype 16/6) of anti-DNA antibodies and (ii) polyclonal IgG and IgM aCL antibodies derived from serum of a patient with primary anti-phospholipid syndrome. After infusion of either antibody (10 μ g per mouse) we could demonstrate lower fecundity rate, increased resorption index of embryos (equivalent to recurrent fetal loss), lower number of embryos per pregnancy, and lower mean weights of embryos and placentae in comparison to mice infused with appropriate control immunoglobulins. We conclude that the aCL antibodies may have direct effects on fecundity and on the outcome of pregnancy.

The "lupus anticoagulant" is an acquired autoantibody that acts by inhibiting the generation of the prothrombin activator complex. A strong correlation has been demonstrated (1, 2) between the lupus anticoagulant and increased anti-cardiolipin levels. It was found that antibodies directed against negatively charged phospholipids—cardiolipin, in particular—could be demonstrated in sera from patients with positive lupus anticoagulant tests. There appears to be a close, but not absolute, correlation between the lupus anticoagulant test and anti-cardiolipin (aCL) antibodies, and there are indications that the antibodies responsible for the lupus anticoagulant test and those reactive with cardiolipin are similar but not identical (1–8).

A paradoxical association between the *in vitro* lupus anticoagulant test and thromboses is known (1). The early association with thrombosis has been confirmed in large groups of patients (3–8). Similarly, the relationship to recurrent fetal loss, presumably resulting from placental thrombosis and infarction, has also been highlighted.

In only two circumstances other than defined systemic lupus erythematosus (SLE), however, do the same associations frequently occur, (i) in "lupus-like" disease, patients who fulfill less than four of the criteria for the classification of SLE, as laid down by the American Rheumatic Association (ARA) (9), and (ii) in primary anti-phospholipid syn-

drome (PAPS), patients recently defined and documented who do not have any of the major clinical or serological features of SLE (10–12).

Whether the anti-phospholipid antibodies constitute "markers" or epiphenomena seen in particular "subsets" of patients with the associated clinical syndromes or whether they are in themselves pathogenic is presently unclear (13).

The aim of the current study was, after passive transfer, to evaluate the pathogenic effect of aCL antibodies from two sources on the induction of PAPS in naive mice and to determine the influence of such passive transfer on fetal loss (14–17).

MATERIALS AND METHODS

aCL Antibodies. CAM, a mouse IgG monoclonal antibody, was produced by a hybridoma generated when a non-secreting myeloma cell line was fused with a splenocyte from a mouse with experimental SLE (18, 19). The latter is induced by immunization with a pathogenic anti-DNA idotype (Id) (16/6 Id). The CAM antibody was derived from BALB/c mouse in which SLE was induced by the MIV-7 antibody (20). MIV-7 is a human monoclonal anti-DNA antibody carrying the 16/6 Id that was produced by the human hybridoma derived from the fusion of the GM-4672 myeloma cell line with *in vitro*-immunized normal human peripheral lymphocytes (21) with an anti-Id antibody to anti-mouse mammary tumor virus (B₁₁) (20, 21). Four months after immunization in the footpad with 1 μ g of MIV-7 in adjuvant and a booster injection of 1 μ g of the antibody in phosphate-buffered saline (PBS), the BALB/c mice developed SLE, including increased erythrocyte sedimentation rate, leukopenia, proteinuria, anti-double-stranded DNA, aCL antibodies, and anti-histone antibodies. Electron microscopy of the kidneys showed 16/6 Id-positive deposits. In addition these mice developed thrombocytopenia and prolonged activated partial thromboplastin time (APTT) (Table 1). The CAM antibody also binds DNA and is 16/6 Id-positive. By itself the CAM antibody is not a lupus anticoagulant.

YD_{G.M.} Y.D. is a 23-year-old female patient with the PAPS. From the age of 16, she had a positive Venereal Disease Research Laboratories (VDRL) test, prolonged partial thromboplastin time, and recurrent deep thrombophlebitis in the legs (three episodes). aCL antibodies of the IgG and IgM isotypes were detected in her serum. These antibodies were isolated by ammonium sulfate precipitation and extensive absorption on goat anti-human IgG- and IgM-Sepharose, respectively. After elution with 5 M MgCl₂ and repeated dialyses against PBS, the IgG and IgM were examined for their binding to cardiolipin, phosphatidylserine, single-stranded DNA, and double-stranded DNA by ELISA.

Table 1. Mice immunized in their footpads with MIV-7

Monoclonal antibody	aCL antibody	Platelet count, no./ml	APTT, sec
MIV-7	+	254,000 \pm 41,000	94 \pm 8
MOBD-5	—	981,000 \pm 55,000	34 \pm 4

MIV-7 is a human monoclonal anti-DNA IgM carrying 16/6 Id. MOBD-5 is a control human monoclonal IgM that does not bind to DNA or to cardiolipin and is negative for 16/6 Id.

Blood Cell Counts and Coagulation Studies. Leukocytes and platelets from individual blood samples were counted on an ELT8/WS cell counter (Coulter). The anticoagulant activity was evaluated by a mixing APTT with cephalin (IL test 97584-20, Instrumentation Laboratory, Lexington, MA) as follows. Plasma was separated from the mouse's blood by centrifugation, added at a ratio of 1:1 (vol/vol) to cephaline, and incubated for 2 min at 37°C. Then, an equal volume of 0.025 M CaCl₂ was added, and the clotting time was recorded. A correction of the APTT was made by exchanging 50% of the plasma tested with normal mouse plasma.

ELISA. Murine anti-phospholipid antibodies in the immunized and pregnant mice were detected by ELISA as follows: 96-well ELISA plates were coated with cardiolipin (50 μ g/ml in ethanol). The plates were dried overnight at 40°C and then blocked for 2 hr with 5% (vol/vol) bovine serum. The mouse sera were diluted 1:200–1:3600 in 2% bovine serum/Tris-buffered saline (TBS). After overnight incubation at 40°C, a 1:1000 dilution of alkaline phosphatase-conjugated anti-mouse IgG in 2% bovine serum was added for 4 hr at room temperature with shaking. The reaction was developed with *p*-nitrophenyl phosphate as substrate and A₄₀₅ was read.

Antibodies to double-stranded DNA and single-stranded DNA were detected using similar methods except that the plates were sequentially coated with poly(L-lysine) (50 μ g/ml), the antigen (2.5 μ g/ml) in question in TBS, and then poly(L-glutamate) (50 μ g/ml). The rest of the assay was similar to aCL antibody detection.

Mice. BALB/c female mice aged 8 weeks, ICR females aged 12 weeks, and ICR males aged 18 weeks were purchased from Tel-Aviv University. The mice were immunohistochemically studied for serological markers (e.g., aCL or anti-DNA antibodies) and pregnant mice were studied for the fate of the litters after immunoglobulin infusion.

Evaluation of Pregnancy Outcome. The number of vaginal plugs (indicating mating in 92% of the mice), the number of live pups per pregnancy, and the weights of embryos and placentae were studied. Elution of possible absorbed or bound aCL antibodies in the placenta was carried out as follows. A whole placenta was broken into minute pieces by passing it through a 10-ml syringe and was washed extensively with PBS, until the supernatant contained no blood. Tissue was mixed with 0.1 M glycine hydrochloride (pH 2.4), and after centrifugation, the supernatant was neutralized by dialysis against PBS. The presence of aCL antibodies and the 16/6 Id in the supernatant was studied by ELISA using plates coated with cardiolipin and rabbit anti-16/6 antibody.

Passive Transfer of aCL Antibodies. A group of 10–15 ICR mice (12 weeks of age) were infused with the various aCL antibodies (10 μ g per mouse) through the tail vein. In parallel, other groups were given a mouse monoclonal antibody (10 μ g per mouse) that does not bind to cardiolipin and human IgG and IgM prepared from a normal subject exactly as the aCL antibodies.

One day after infusion the mice were mated. The number of vaginal plugs, indicating mating, was counted every morning. The antibody CAM was injected at days 0 (mating), 4, 9, and 16 of pregnancy. Antibodies YD_G, M were injected at days –1 and 4 only.

The 16/6 Id titer of isolated immunoglobulins or serum was determined as described (18).

RESULTS

Two groups of mice were infused with CAM antibody (10 μ g per mouse) (an aCL antibody) and N-40, a mouse monoclonal IgG (10 μ g per mouse) that was generated from the same fusion yet lacked aCL binding. Table 2 summarizes the outcome of pregnancy in the two groups of mice. The rate of resorptions, calculated as the number of resorped fetuses (*R*) divided by resorped and full-term fetuses (*F*) [$\%R = R/(R + F)$], was 68 \pm 7% in the mice given 10 μ g of the CAM antibody. The ICR mice being a non-outbred species and having high fertility rates had no resorptions out of 12 pregnancies in each group. No significant differences were found in the rate of fecundity between the groups. The pregnant mice infused with the CAM antibody had thrombocytopenia, prolonged APTT, and significantly smaller placentae and embryos in comparison to mice injected with the control immunoglobulins or placebo (Table 2). These findings are shown in Fig. 1.

Since it seems logical that the CAM antibody (16/6 Id+) is absorbed in the placenta, we measured the decline in 16/6 Id titer in sera of mice transfused with CAM and control antibodies. As can be seen from Fig. 2A, after transfusion of CAM, nonpregnant mice had only a small decline in 16/6 Id titer after 18 days. In contrast, a 60% decline in serum 16/6 Id level was recorded in pregnant mice after 18 days. Eluted immunoglobulins from placentae of pregnant mice injected with CAM showed aCL activity and were 16/6 Id-positive, whereas the immunoglobulins eluted from control placentae were negative for aCL and 16/6 Id. The A₄₀₅ units ($\times 10^3$) for 16/6 Id levels and aCL antibodies were 847 \pm 74 and 783 \pm 81 for mice injected with CAM, respectively, and 24 \pm 3 and 32 \pm 7 for mice given control immunoglobulin N-40.

Fig. 2B shows the effect of injecting the aCL antibody CAM at various stages of pregnancy. Increased resorption rates after CAM infusion were noted when the antibody was injected during the first third of the pregnancy and declined significantly toward day 16.

In the second experiment eight groups of 15 ICR mice were infused in the tail vein with YD_G, YD_M, or IgG and IgM controls at 10 μ g per mouse 1 day before (day –1) and 4 days after mating. The mice were sacrificed on day 18 of pregnancy. Table 3 summarizes the number of vaginal plugs, number of

Table 2. Effects of aCL antibody on pregnancy

Monoclonal antibody	% resorption	Platelet count, no./ml	APTT, sec	Placenta weight, mg	Embryo weight, mg
CAM	68 \pm 7	268,000 \pm 12,000	83 \pm 7	143 \pm 8	790 \pm 110
N-40	1 \pm 0.02	842,000 \pm 38,000	48 \pm 5	179 \pm 9	1410 \pm 220
None (PBS)	0	1,123 \pm 229,000	46 \pm 6	183 \pm 11	1520 \pm 180

Mice were transfused at day 0 (day of mating) with mouse monoclonal aCL antibody CAM, control monoclonal immunoglobulin (N-40), or a placebo (PBS). The APTT value of mice before pregnancy was 31 \pm 4 sec and the platelet count was 994,000 \pm 89,000 platelets per ml. Pregnant mice were sacrificed on day 19.

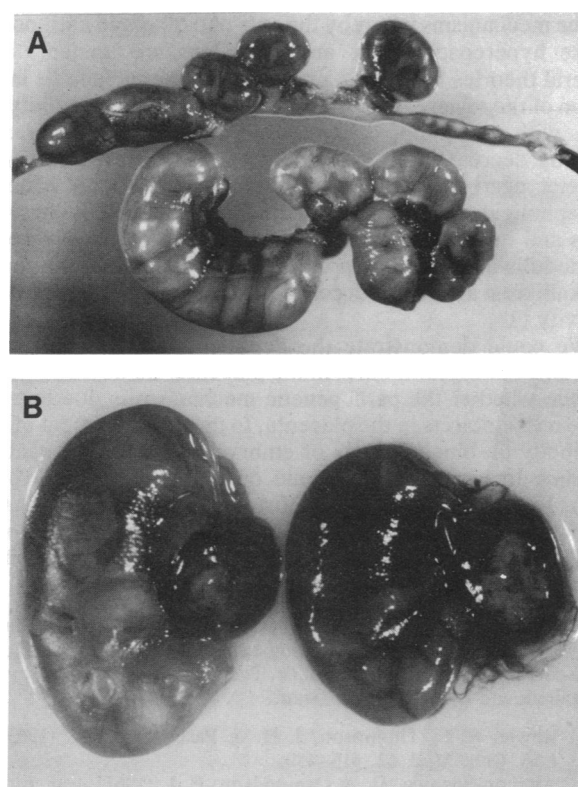


FIG. 1. (A) Upper uterus with placenta and fetuses is representative of a mouse infused with CAM aCL antibody on the day of mating (day 0). Four small fetuses can be seen with several resorpted fetuses (with patches). Lower uterus was derived from a mouse infused with the normal control immunoglobulin (N-40). (B) Isolated amnion sacs with fetuses (18 days of gestation). The sac from the CAM-infused mouse to the left has a smaller placenta with hemorrhages. The sac from the N-40-infused mouse to the right has a normal placenta and fetus.

pregnant mice, the mean \pm SD of weights of the embryos and placentae, and platelet number and APTT in the mice injected 4 days after mating. As can be seen the number of mice with plugs and number of pregnant mice were significantly lower in the YD_G-infused mice in comparison to controls.

In addition the mean weights of embryos and placentae (IgG-infused only) were significantly ($P < 0.001$) smaller than those of controls (Fig. 3). These effects were less impressive with the aCL IgM (YD_M).

Table 3. Mice infused with human IgG and IgM aCL

Monoclonal antibody administered	No. mice with plugs/ no. mice mated	No. pregnant mice/ no. mice mated	Embryo weight, mg	Placenta weight, mg	Platelet count, no./ml	APTT, sec
YG _a						
Day -1	3*/15	3/15	693 \pm 92	131 \pm 34	ND	ND
Day 4	10/10	5/10	—	—	380,000 \pm 55,100	102 \pm 9.8
NG _a						
Day -1	6/14	6/14	1157 \pm 168	175 \pm 48	ND	ND
Day 4	10/10	10/10	1155 \pm 148	156 \pm 1	1,316,000 \pm 55,100	34 \pm 2
YD _M						
Day -1	7/15	6/15	846 \pm 147	151 \pm 35	ND	ND
Day 4	10/10	9/10	835 \pm 103	139 \pm 12	1,406,000 \pm 128,600	49 \pm 2
NC _M						
Day -1	9/14	9/14	1102 \pm 121	151 \pm 37	ND	ND
Day 4	10/10	9/10	1123 \pm 148	149 \pm 7	1,436,000 \pm 40,400	31 \pm 3

ND, not done.

*In one out of the three mice with plugs, 12 pups were born on day 16. In a second mouse, a 100% resorption index was noted (Fig. 3). In a third mouse, 10 embryos were noted with one resorption.

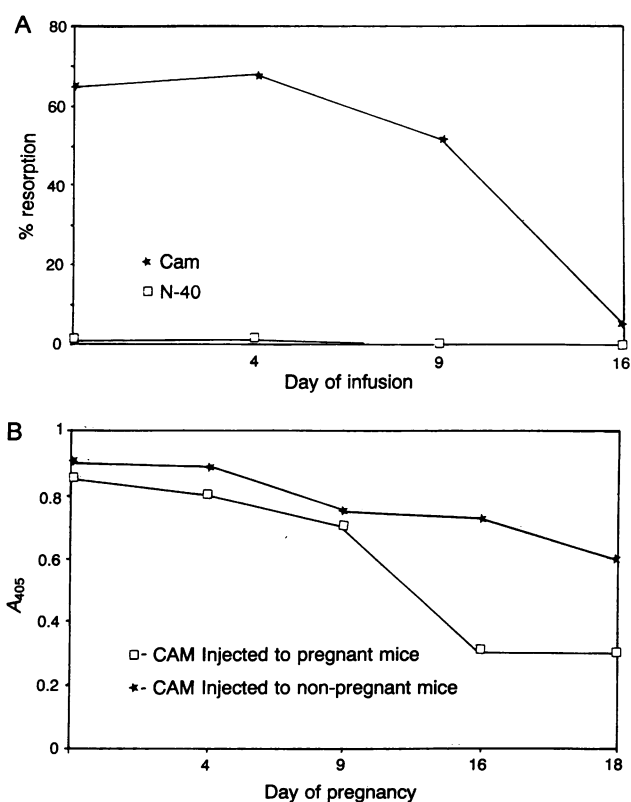


FIG. 2. (A) Rate of resorption of fetuses in ICR mice transfused with aCL antibody CAM and control immunoglobulin on days 0, 4, 9, and 16. (B) 16/6 Id level in sera of pregnant and nonpregnant mice injected with CAM (16/6 Id+) antibody recorded on days 0, 4, 9, 16, and 18. In pregnant mice a decline in 16/6 Id antibody titers is noted presumably due to absorption to the placenta. Immunoglobulins eluted from placenta were found to react with cardiolipin ($OD \times 10^3$, 783 ± 81) and to carry the 16/6 Id ($OD \times 10^3$, 847 ± 74) whereas control immunoglobulins were negative (32 ± 7 and 24 ± 3 , respectively).

The most significant effect after aCL IgG injection was noted on the weights of embryos and placentae (mean \pm SD), whether the antibody was given on day -1 or day 4. Interestingly, in all five pregnant mice injected with YD_G on day 4 after plug appearance, 100% resorption was noted. In parallel, the mice injected with the aCL IgG developed thrombocytopenia and prolonged APTT, indicating the presence of the anti-phospholipid syndrome.

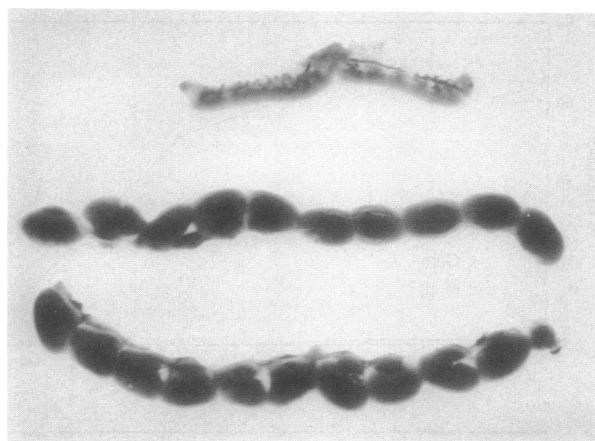


FIG. 3. Uterus of mice immunized with YD_G aCL antibody showing 100% resorption of fetuses in one mouse (Top) and smaller embryos in another mouse (Middle) whereas in mice infused with control human immunoglobulin normal-size embryos can be seen (Bottom).

DISCUSSION

PAPS can be diagnosed in a young person (<45 years) with any of the following symptoms: more than one episode of unexplained venous thrombosis, one or more episodes of arterial thrombotic events (without risk factors), one or more pregnancy losses, and high titers of IgG aCL, and an unequivocally positive lupus anticoagulant test (22).

It has since become clear that aCL antibodies may be demonstrable in a variety of conditions that include other "autoimmune" diseases (23–29) and infections, particularly viral and spirochetal infections (30). They may also be induced by certain drugs such as chlorpromazine and procainamide (31–33).

There is an association between the presence of aCL antibodies and the occurrence of repeated spontaneous abortions (14–17). Yet, most of the women with aCL antibodies do not suffer from recurrent abortions. Therefore, the only way to confirm the pathogenic role of aCL antibodies in pregnancy is to repeat the classical studies in autoimmune diseases, namely, demonstrating the induction of clinical signs and symptoms after passive transfer of the pathogenic antibody. In this study we evaluated the effect of passive transfer of aCL antibodies derived from two sources, on the outcome of pregnancy in ICR mice. These mice are an outbred strain with high rate of fecundity and high number of litters. The mouse monoclonal aCL antibody CAM was derived from a mouse with experimental SLE induced by a pathogenic anti-DNA idotype (18). In this model, SLE was induced in naive mice by immunization with a pathogenic anti-DNA Id (16/6 Id). After ID dysregulation, the mouse developed anti-DNA antibodies carrying the 16/6 Id. After 4 months, serological and clinical manifestations of SLE could be seen. The latter include leukopenia, proteinuria, increased sedimentation rate, raised levels of anti-DNA and anti-La antibodies, etc., and deposition of immunoglobulins carrying the 16/6 Id in the kidneys. Mice immunized with MIV-7, in addition to aCL antibodies, had thrombocytopenia, prolonged APTT, and serological markers of the PAPS. The human MIV-7 aCL antibodies were derived from a patient with a PAPS.

In both occasions, passive transfer of the purified aCL antibodies was associated with lower fecundity or/and an increased rate of embryo absorptions [equivalent to primary infertility (34) and recurrent abortions reported in humans with PAPS (14–16)] and smaller litters and placentae, in comparison to mice infused with the control IgG.

The mechanisms whereby the anti-phospholipid antibodies cause hypercoagulability and fetal loss are unclear, but several theories have been proposed. These include (i) inhibition of the release of arachidonic acid by cross-reactivity of aCL with phospholipid in the endothelial cell membrane, thereby decreasing prostacyclin production and enhancing platelet aggregation (35), (ii) cross-reaction with platelet phospholipids, causing damage that increases their adhesiveness and initiates thrombosis (36), (iii) inhibition of thrombomodulin activation by protein C (37), and (iv) inhibition of prekallikrein activity with consequent decrease in fibrinolytic activity (38).

We could demonstrate the deposition of the transfused (16/6 Id+) aCL antibodies in the placenta. So far, we cannot decide whether the pathogenetic mechanism is due to multiple microinfarcts in the placenta, to direct toxic effect of the antibody on blood vessels or embryos, or to cross-reactive binding to luteinizing hormone or chorionic gonadotropin (39). However, our study confirms the importance of aCL antibodies in induction of fetal loss. This experimental model of PAPS may enable studies of better preventive measurements of fetal loss and thromboembolic phenomena.

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